An Adenovirus Type 5 Early Region 1B-Encoded CTL Epitope-Mediating Tumor Eradication by CTL Clones Is Down-Modulated by an Activated *ras* Oncogene[1]

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Mouse embryo cells (C57BL/6, H-2^b) transformed by the E1A and E1B genes of adenovirus type 5 (Ad5E1 MEC) are highly immunogenic. Previously, CTL were cloned from mice immunized with Ad5E1 MEC. These CTL clones were capable of tumor eradication in nude mice, and were directed against the Ad5E1A-encoded decapeptide SGPSNTPPEI, presented by the H-2D^b MHC molecule. We have now generated Ad5E1 MEC containing a mutated Ad5E1A-encoded epitope. The mutant Ad5E1 MEC induce a strong CTL response when injected into immunocompetent mice. CTL clones generated against mutant Ad5E1-transformed tumor cells recognize an Ad5E1B-encoded epitope (VNIRNCCYI) in the context of H-2D^b. Because this epitope is also present on wild-type Ad5E1 MEC, it is concluded that Ad5E1-transformed tumor cells express at least two CTL epitopes. Interestingly, the lysis of Ad5E1 MEC by the Ad5E1B-specific, but not by the Ad5E1A-specific, CTL clones was strongly diminished by the action of the activated *ras* oncogene. CTL directed against the Ad5E1B-encoded epitope were, like Ad5E1A-specific CTL, able to eradicate large established Ad5E1-induced tumors in B6 nude mice, demonstrating that CTL activity directed against different CTL epitopes expressed by the same tumor can be exploited for immunotherapy of cancer. *The Journal of Immunology,* 1995, 154: 3396–3405.

Tumors induced by viruses often express Ags that can induce a T cell-mediated tumor-specific immune response (1–3). Tumor Ags responsible for the generation of tumor-specific T cells against virus-induced tumors are generally found to be encoded by viral genes (for review, see Refs. 1, 3). For instance, the T cell response of C57BL/6 (H-2^b) mice (B6 mice) against a Friend leukemia virus-induced FBL-3 tumor was shown to be aimed at viral envelope and gag components (4). The immunodominant CTL response of C57BL/10 (H-2^b) mice (B10 mice) against mink cell focus-inducing murine leukemia virus-induced B10 tumor cells is directed against viral envelope protein (5). CTL generated against SV40 transformed cells in B6 mice recognize peptides encoded by the viral T Ag (6–8).

Tumors induced by human adenovirus type 5 (Ad5) can be eradicated by adoptive transfer of cloned CD8$^+$ CTL (for review see Refs. 1, 2, 9). In the case of Ad5 early region 1- (Ad5E1-)[4] induced tumors, a peptide recognized by the CTL was shown to be encoded by the viral E1A oncogene (10). This peptide, with the sequence SGPSNT PPEI, is recognized in the context of the H-2D^b MHC molecule (11). An alternative approach for the prevention and treatment of cancers is immunization with T cell epitope-containing synthetic peptides. Recently, we showed that immunization with a synthetic peptide deduced from the early-region 7 (E7) gene product of the human papilloma virus type 16 (HPV 16) led to the induction of peptide-specific CTL that recognized HPV 16-induced tumors. Moreover, peptide-vaccinated mice were protected against tumor

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[4] Abbreviations used in this paper: Ad5E1A, adenovirus type 5 early region 1A; Ad5E1B, adenovirus type 5 early region 1B; HPV, human papilloma virus; MEC, mouse embryo cells; WT, wild-type.

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outgrowth after a challenge with a lethal dose of HPV 16-induced tumor cells (12).

A potential drawback of adoptive T cell transfer or any other form of T cell immunotherapy is tumor cell escape by alteration or down-modulation of the relevant T cell epitope. Several investigators have shown that tumor cell variants escaping CTL recognition can emerge during cocultivation of tumor cells with tumor-specific CTL (8, 13–15). In those cases, the escape variants of the tumor cell contained mutations in the CTL epitope-coding sequences (15–17). A possible approach to minimize the risk to select for tumor Ag-negative variants during T cell-based immunotherapy is to direct the T cell response toward different T cell epitopes expressed by the same tumor. Such an approach should be feasible in the case of, for example, melanoma, because several different tumor Ags expressed by melanoma cells have been reported (18–24).

The purpose of this study was to determine whether a tumor expressing two distinct T cell epitopes can be eradicated by adoptive transfer of CTL clones directed against either one of the epitopes. It is demonstrated that Ad5E1-induced tumors are indeed eliminated in vivo by both Ad5E1A- and Ad5E1B-specific CTL. Our data also show that the capacity of the Ad5E1B-specific CTL to lyse Ad5E1-expressing tumor cells is compromised if these tumors express, in addition to Ad5E1, an activated E*Jras* oncogene, indicating that the recognition of the Ad5E1B-encoded CTL epitope is down-modulated by the action of the E*Jras* oncogene.

Materials and Methods

Mice

C57BL/6 (B6 Kb, H-2^b) mice were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands) and C57BL/6 *nu/nu* (B6 nude, H-2^b) were obtained from Bomholtgard (Ry, Denmark).

Cell lines and culture conditions

Ad5E1A genes with mutations in the region encoding the peptide SGPSNTPPEI were generated as follows: The E1A gene was excised as a *HindIII-PstI* fragment from the plasmid p*Ad5PstI* (25) and cloned into pUC13. The resulting plasmid, pUCAd*5PstI*, was digested with restriction endonuclease DraIII, which cuts at positions 953 and 1410 within the Ad5 sequence (26). The large restriction fragment, which was separated from the 953–1410 DraIII fragment by electrophoresis, was used as a vector. Into this vector DraIII fragments were ligated that were generated by PCR, using pUCAd*5PstI* as a template and as primers the following oligonucleotides: T→I, upstream primer 5′-GGC.TTT.CCA.CCC.AGT.GAC.GAC.GAG-3′, downstream primer 5′-GGA.CCA.CCG.GGT.GTA.TCT.CAG.GAG.GTA.TGT.TAG-3′; P→S, upstream primer 5′-GGC.TTT.CCA.CCC.AGT.GAC.GAC.GAC.GAG-3′, downstream primer 5′-CGG.GAC.CAC.CGG.GTG.TAT.CTC.AGA.-AGG.TGT-3′ (underlined: DraIII-recognition sequence; underlined and bold: mutation in peptide-coding region). The resulting mutant-Ad5E1A constructs were sequenced across the 953–1410 DraIII-fragments in two directions to assure the presence of the desired mutations as well as the absence of additional mutations that could have been introduced during the PCR reaction. B6 mouse embryo cells (B6 MEC) expressing either mutant (T→I; P→S) or wild-type (WT) Ad5E1A were generated by transfection of primary MEC with an E1A-encoding plasmid together with pTK-neo (10) and pSV-Ad5E1B (pPDC11, 27). Expression of the E1A and E1B gene products in the transformed MEC was verified both at the mRNA (Northern blotting) and protein level (immunoprecipitation) (data not shown).

Cells expressing Ad5E1 and E*Jras* were generated by transfection with p*Ad5XhoIC* (28), p*EJras* (29), and pTK-neo (10). Expression of Ad5E1A, Ad5E1B, and E*Jras* gene products was confirmed both at the mRNA and protein level (data not shown). Supertransfection of Ad5E1(wt) MEC and Ad5E1(P→S) MEC with *EJras* and/or pTK-hygro was done by transfection with p*EJras* and pTK-hygro. The latter plasmid contains the HSV-tk promoter in front of the hygromycine resistance gene. All other cell lines used were generated as previously described (10). All cells were maintained as described elsewhere (10, 30).

Peptides

"Bulk" peptides were generated by solid-phase strategies on a multiple peptide synthesizer (Abimed AMS 422) as described previously (12, 31). Synthetic peptides tested for recognition by CTL clones generated against mutant Ad5E1 MEC are: SGPSNTPPEI, SGPSNIPPEI, SGPSNTPSEI, MERRNPSERGV, PPGDNTDGGAA, PSGMNVVQVA, EEARNLAFSLM, QIKDNCANEL, DCCANELDLLA, VNIRNCCYI, YISGNGAEVEI, CSMINMWPGVL, FYGPNFSGTVF, VFLANTNLII, LANTNLILHGV, FYGFNNTCVEA, YGFNNTCVEA, MVCGNCEDRA, CSDGNCHLI, LHLGNRRGVFL, PYQCNLSHTKI, MSKVNLNGVF, KVNLNGVFDM, KHIRNQPVML.

Peptide binding studies and FACS analysis

Peptide binding studies and FACS analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) were performed as described elsewhere (12).

Cell-mediated lymphocyte cytotoxicity

Experimental procedures to measure cell-mediated cytotoxicity were performed in a Europium-(Eu^{3+}) release assay as described elsewhere (12, 32). In short, varying numbers of effector cells were added to 2×10^3 Eu^{3+}-labeled target cells in 0.15 ml of culture medium in 96-well U-bottom plates. After a 4-h incubation at 37°C, supernatants were collected and mixed with Enhancer solution (Wallac, Turku, Finland). Measurement of the samples took place in a 1234 Delfia fluorometer (Wallac). The mean percentage specific lysis of triplicate wells was calculated as follows: % Specific lysis = [(cpm experimental release − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)] × 100.

Generation of tumor-specific CTL clones

Tumor-specific CTL in bulk culture and CTL clones were generated against WT and mutant Ad5E1-transformed cells according the methods described previously (10, 33). CTL clones were routinely stimulated once a week, and maintained as previously described (10). All CTL clones and lines obtained had the marker profile Thy-1$^+$, CD4$^-$ and CD8$^+$.

Transfection of COS-7 cells

Transient transfection in COS-7 cells was performed as described elsewhere (34). In short, 100 ng of plasmids p*Ad5XhoIC*, p*Ad5/12*, p*Ad12/5*, p*Ad12EcoRIC*, p*Ad5PstI*, pPDC11, p*5dlSac*, or p5XT together with 100 ng of plasmid pcDNAI/Amp-D^b were transfected by the DEAE-dextran-chloroquine method into 1×10^4 COS-7 cells (35). Plasmid p*AdXhoIC* harbors the Ad5E1A and E1B genes; p*Ad5/12*, p*Ad12/5* and p*Ad12EcoRI* harbor respectively: Ad5E1A/Ad12E1B, Ad12E1A/5E1B, and Ad12E1A/12E1B (28). p*Ad5PstI* (25) and pPDC11 harbor respectively only Ad5E1A and Ad5E1B (27). p*5dlSac* harbors Ad5E1A/Ad5E1B 55 kDa (not Ad5E1B 21 kDa), whereas p5XT harbors Ad5E1A/Ad5E1B 21 kDa (not Ad5E1B 55 kDa) (36, 37). Plasmid pcDNA1/Amp-D^b contains the H-2D^b gene. The plasmid pcDNAI-D^b was constructed by ligating the *BamHI/XbaI* and *XbaI/PvuII*-fragments of a genomic H-2D^b clone (exons 1–3 and 5–8 respectively (38) into pcDNAI/Amp (Invitrogen, San Diego, CA). The COS cells were incubated in 100 ml Iscove's modified Dulbecco's medium containing 8% FCS for 72 h at 37°C, after which 1500 CTL in 25 ml Iscove's modified Dulbecco's medium containing 50 Cetus Units of recombinant IL-2 (rIL-2, Cetus Corp., Emeryville, CA) were added. After 24 h, the supernatant was collected and its TNF content was determined by measuring its cytotoxic effect on WEHI-164 clone 13 cells as previously described (30).

In vivo administration of tumor-specific CTL clones

In vivo therapy for Ad5E1-induced tumors with tumor-specific CTL clones was performed as described previously (10), with slight modifications. In short, B6 *nu/nu* mice with Ad5E1-induced tumors ranging from 40 to 50 mm^3 were treated with i.v. injections of B6 Ad5E1A-specific CTL clone 5 (1.5×10^7)($n = 5$), B6 CTL clone 01 generated against B6 Ad5E1(T→I) cells (1.5×10^7)($n = 5$), or B6 CTL clone 21 generated against B6 Ad5E1(P→S) cells (1.5×10^7)($n = 5$) in combination with 2.5×10^3 Cetus Units rIL-2, administered s.c. in IFA at a site distant from the tumor. The s.c. rIL-2 administration was repeated weekly. Animals in control groups ($n = 5$) were killed when their tumors grew larger than 500 mm^3 to avoid unnecessary suffering.

Results

Generation of Ad5E1 MEC with an altered Ad5E1A-encoded CTL epitope

In previous studies the optimal size of the peptide recognized by Ad5E1A-specific CTL clones for binding to H-2D^b-molecules and for recognition by these CTL clones was shown to be ten amino acids (sequence SGPSNTP PEI) (10, 11). This amino acid sequence lies outside the Ad5E1A domains involved in neoplastic transformation, allowing replacement of amino acids at this site without changing the transforming capacity of the Ad5E1A-region (39, 40). Through an amino acid replacement analysis using synthetic peptides, the amino acids in this peptide important for recognition by Ad5E1A-specific CTL clones were identified (11, Kast, W.M., A.R. Offring, and C.J.M Melief, unpublished observations). The peptides SGPSNI PPEI (T→I) and SGPSNTPSEI (P→S) were selected for further analysis. Both peptides bind to H-2D^b-molecules with comparable affinity as the WT Ad5E1A decapeptide (Fig. 1*A*), but the P→S peptide is recognized 70 times less efficiently by Ad5E1A-specific CTL clone 5 (10) compared with the T→I peptide or the WT peptide (Fig. 1*B*).

On the basis of these studies, Ad5E1 MEC were generated, which express Ad5E1A-genes harboring these mutations in the Ad5E1A-encoded CTL epitope. The recognition of these tumor cell lines by Ad5E1A-specific CTL clone 5 follows the prediction of the amino acid replacement analysis as performed with synthetic peptides. The tumor cell lines expressing the T→I mutation (Ad5E1 (T→I) MEC) are efficiently recognized by Ad5E1A-specific CTL clone 5, whereas the tumor cell lines expressing the P→S mutation (Ad5E1(P→S) MEC) are not recognized by Ad5E1A-specific CTL (Fig. 2).

CTL clones generated against (mutant) Ad5E1 MEC

To determine the effects of these mutations on the immunogenicity of Ad5E1-transformed tumor cells, the CTL activity induced by immunization with the tumor cell lines was tested. Bulk CTL derived from mice immunized with either WT or mutant Ad5E1-transformed MEC displayed high tumor-specific CTL activity (data not shown). CTL clones were isolated from CTL bulk cultures to identify

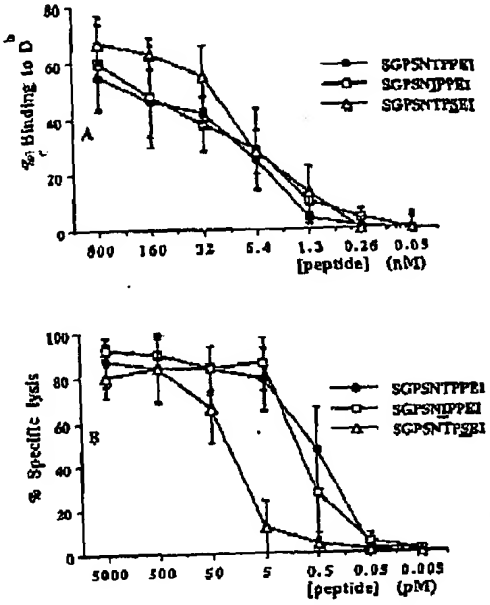

FIGURE 1. Binding of WT Ad5E1A peptide$_{234-243}$ SG PSNTPPEI, mutant peptide SGPSNIPPEI and mutant peptide SGPSNTPSEI to H-2D^b-molecules of RMA-S (*A*), and recognition of these peptides by B6 Ad5E1A-specific CTL clone 5 (*B*). *A*) The RMA-S MHC class I-peptide binding assay was performed as previously described (12). Cells were stained with mAb specific for H-2D^b (41) and a fluorescein isothiocyanate-labeled second Ab. The percentage of binding to D^b was calculated by the formula: % of binding to D^b = 100 × [(mean fluoresence experimental well − mean fluoresence background)/(mean fluoresence WT peptide SGPSNTPPEI at a concentration of 100 μM − mean fluoresence background)]. Mean values ± SD of four experiments are shown. *B*) Recognition of syngeneic EL-4 cells by B6 Ad5E1A-specific CTL-clone 5 incubated with WT and mutant peptides in 10-fold dilutions. The mean percentage of specific lysis as determined by an Eu^{3+}-release cytotoxicity assay ± SD of five experiments by an effector-to-target cell ratio of 10 is shown.

the nature of the CTL-epitope(s) presented by the (mutant) Ad5E1-transformed cells.

CTL clone 42, which was raised against Ad5E1(wt) MEC, lysed Ad5E1(wt) MEC and Ad5E1(T→I) MEC, whereas untransformed MEC and Ad5E1(P→S) MEC were not lysed (Fig. 3*A*). CTL clone 42 recognized WT, T→I, and P→S decapeptides at the concentration used in the cytotoxicity assay. Thus, CTL clone 42 shows the same specificity pattern as CTL clone 5 and other previously described Ad5E1A-specific CTL clones (10, 11).

CTL clones raised against Ad5E1(T→I) MEC and Ad5E1(P→S) MEC displayed a different recognition profile. These CTL, although lysing both mutant- and WT

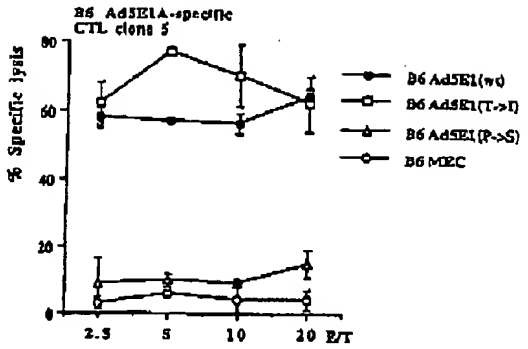

FIGURE 2. Specific lyis of untransformed B6 MEC and B6 Ad5E1-transformed tumor cell lines expressing a WT or mutant Ad5E1A-encoded epitope by B6 Ad5E1A-specific CTL clone 5 as determined by an Eu^{3+}-release cytotoxicity assay. Target cells: untransformed B6 MEC; B6 Ad5E1(wt), expressing the WT peptide SGPSNTPPEI; B6 Ad5E1(T→I), expressing peptide SGPSN[PPEI) and B6 Ad5E1(P→S) expressing peptide SGPSNTPSEI.

Ad5E1-transformed cells, did not recognize any of the WT Ad5E1A, T→I and P→S peptides (Fig. 3). The lysis of Ad5E1 MEC cannot be attributed to lymphokine-activated killer cell- or NK cell-like activity. First, the recognition of these cells is CD8 mediated, as it can be blocked by anti-CD8 Abs (Fig. 4*A*). Furthermore, these CTL clones are H-2D^b-restricted because they were able to kill Ad5E1-transformed target cells of B6 (K^b, D^b) and 4R (K^k, D^b) origin, but not of 5R (K^b, D^d) origin (Fig. 4*B*). Moreover, anti-H-2D^b Abs, but not anti-H-2K^b Abs were able to inhibit the lysis of B6 Ad5E1 MEC (data not shown). Taken together, these results indicate that Ad5E1(wt) MEC express additional antigenic sites, distinct from the Ad5E1A-encoded peptide SGPSNTPPEI, that are recognized by H-2D^b-restricted CTL clones.

In an attempt to establish the identity of the antigenic peptide recognized by these CTL clones, AdE1A and/or AdE1B containing plasmids were cotransfected into COS cells together with the H-2D^b gene. After 72 h, the transfected COS cells were tested for the expression of the Ag in their ability to cause TNF release by the relevant CTL. The presence of TNF in the culture supernatant was measured by the cytotoxic effect on WEHI-164 clone 13 cells (30). B6 CTL clone 5, directed against Ad5E1A, recognized only COS cells transfected with the H-2D^b gene together with plasmids containing the Ad5E1A-gene (Table I). B6 CTL clone 02, generated against B6 Ad5E1(T→I) MEC, recognized COS cells cotransfected with the H-2D^b gene and plasmids containing the Ad5E1B-gene coding for the 55-kDa Ad5E1B-gene product (Table I). In addition, six other CTL clones directed against mutant Ad5E1 MEC were only stimulated by COS cells transfected with H-2D^b and plasmids coding for the 55-kDa Ad5E1B gene product (data not shown). These results indicate that the

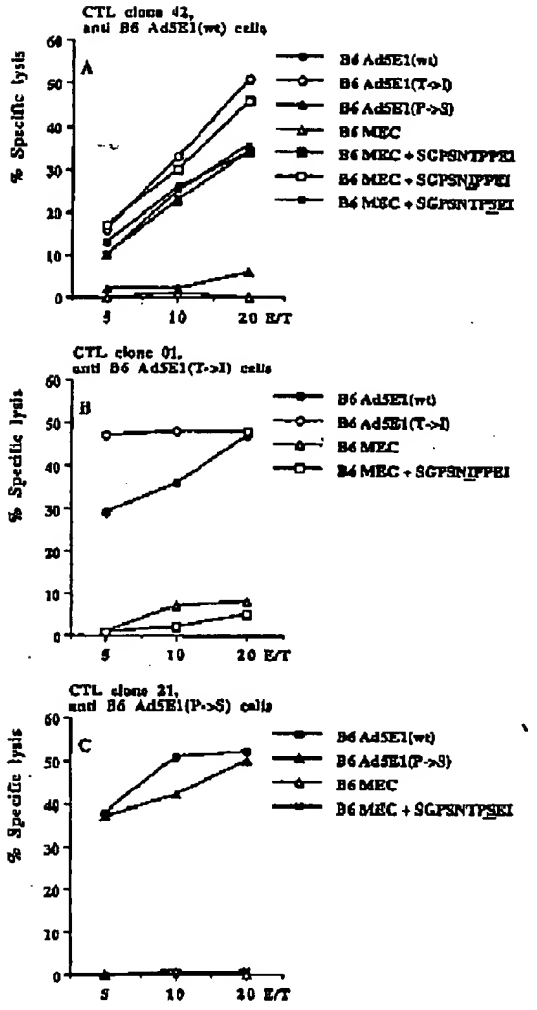

FIGURE 3. Recognition pattern of CTL clones generated against WT and mutant B6 Ad5E1-transformed tumor cells. Lytic activity of B6 CTL clone 42 raised against B6 Ad5E1(wt) MEC (*A*); B6 CTL clone 01, raised against B6 Ad5E1(T→I) MEC (*B*) and B6 CTL clone 21, raised against B6 Ad5E1 (P→S) MEC (*C*) on untransformed B6 MEC (unloaded or peptide loaded (0.5 μM)) and B6 Ad5E1-transformed tumor cells expressing a WT or mutant Ad5E1A-encoded epitope at different effector-to-target cell ratios is shown. Lytic activity was determined by an Eu^{3+}-release cytotoxicity assay. Target cells: untransformed B6 MEC; B6 Ad5E1(wt), expressing the WT peptide SGPSNTPPEI; B6 Ad5E1(T→I), expressing the peptide SGPSN[PPEI, and B6 Ad5E1(P→S) expressing the peptide SGPSNTPSEI.

Ad5E1B 55-kDa gene was responsible for the expression of the Ag recognized by CTL clones generated against mutant Ad5E1 MEC.

To identify the peptide recognized by the Ad5E1B-specific CTL clones, the amino acid sequence of the Ad5E1B

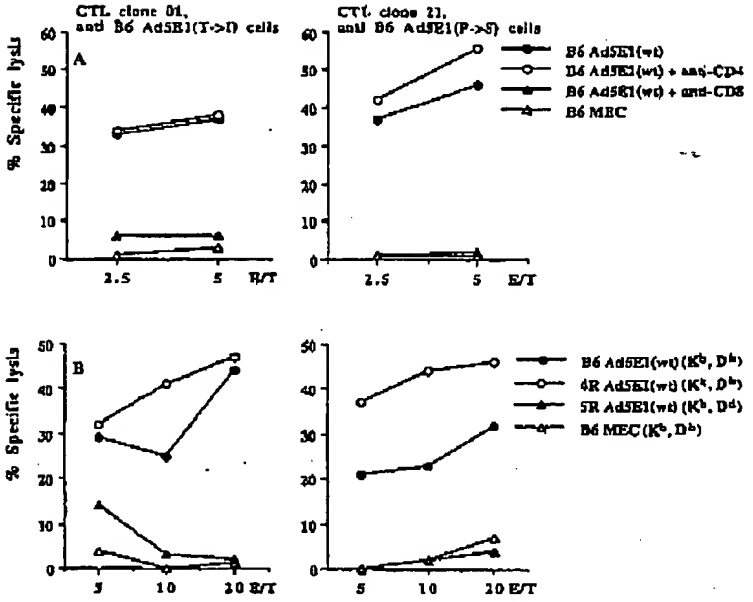

FIGURE 4. Recognition pattern of B6 CTL clones generated against mutant B6 Ad5E1-transformed tumor cells. *A*) Lytic activity of B6 CTL clone 01, raised against B6 Ad5E1(T→I) MEC and B6 CTL clone 21, raised against B6 Ad5E1(P→S) MEC on untransformed B6 MEC and B6 Ad5E1(wt) MEC was tested in the absence or presence of anti-CD4 (42) or anti-CD8 (43) mAb. Lysis of B6 Ad5E1(wt) MEC could be inhibited by anti-CD8, but not by anti-CD4 mAb. *B*) Lytic activity of B6 CTL clones 01 and 21 on untransformed B6 MEC and Ad5E1(wt) transformed tumor cells of various H-2 types. Only Ad5E1-transformed tumor cells expressing H-2D^b are lysed. Percentage of specific lysis as determined by an Eu^{3+}-release cytotoxicity assay at different effector-to-target cell ratios is shown.

Table I. *Stimulation of CTL clone 5 and CTL clone 02 by COS cells transfected with different AdE1A and AdE1B-containing plasmids*[a]

Plasmid	CTL clone 5 anti B6 Ad5E1 (wt)	CTL clone 02 anti B6 Ad5E1 (T→I)
—	0	0
D^b	12	−1
D^b + 5E1A/5E1B	102	102
D^b + 5E1A/12E1B	101	−11
D^b + 12E1A/5E1B	19	102
D^b + 12E1A/12E1B	1	−12
D^b + 5E1A	102	−13
D^b + 5E1B	20	100
D^b + 5E1A/5E1B (55 kD)	103	101
D^b + 5E1A/5E1B (21 kD)	103	15

[a] COS cells transfected with pcDNAI/Amp containing H-2D^b and plasmids harboring different AdE1A and/or E1B genes were used as stimulator cells for CTL clone 5 and CTL clone 02 as described in *Materials and Methods*. Controls were untransfected COS cells or COS cells transfected with only H-2D^b.

Presence of TNF in supernatant upon stimulation of CTL is depicted as % WEHI-164 clone 13 cell death. Percentage of WEHI-164 clone 13 cell death was calculated by the formula: [1 − ($OD_{350-650}$ in sample wells/$OD_{350-650}$ in wells containing untransfected COS cells and CTL)] × 100.

55-kDa protein was screened for the presence of peptide motifs for the H-2D^b-molecule (44, 45), a method successfully applied to identify a *Listeria* monocytogenes epitope (46) and a murine leukemia virus CTL epitope (5). All 21 peptides encoded by Ad5E1B 55 kDa that fulfilled the criteria of the H-2D^b motif (see *Materials and Methods*) were synthesized in bulk quantities and tested for recognition by CTL directed against Ad5E1B. As shown in Fig. 5, one peptide sensitized RMA cells for lysis by CTL clone 21. This 9-mer peptide (sequence: VNIRNCCYI, position 192–200 of the 55-kDa Ad5E1B protein) was also

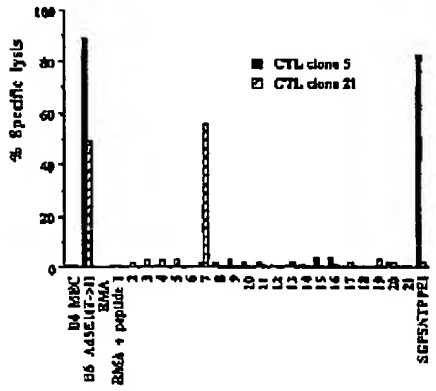

FIGURE 5. Recognition of Ad5E1B 55-kDa$_{192-200}$ encoded peptide VNIRNCCYI by B6 CTL clone 21, generated against B6 Ad5E1 (P→S) cells. Syngeneic RMA cells unloaded or loaded with 21 H-2D^b-motif bearing Ad5E1B 55-kDa encoded peptides (see *Materials and Methods*) or Ad5E1A$_{234-243}$ peptide SGPSNTPPEI were used as target cells for B6 CTL clone 21 and B6 Ad5E1A-specific CTL clone 5 in an Eu^{3+}-release cytotoxicity assay. Peptide concentrations during the experiment were 0.5 μM. Percentage of specific lysis at an effector-to-target cell ratio of 10 is shown.

recognized by CTL clone 21 and five other independently derived CTL clones, generated against mutant Ad5E1 MEC (data not shown). Thus, B6 Ad5E1(wt) transformed tumor cells present, in addition to an Ad5E1A-encoded epitope, a CTL epitope which is derived from the 55 kDa protein of Ad5E1B. This epitope harbors an H-2D^b-specific peptide motif and is recognized by H-2D^b-restricted CTL clones.

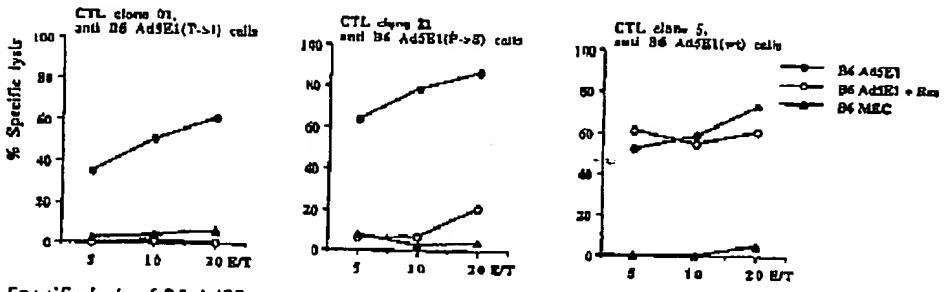

FIGURE 6. Specific lysis of B6 Ad5E1-transformed tumor cells by Ad5E1B-specific CTL clones is abolished by introduction of the activated EJ*ras* oncogene. Lytic activity as determined in an Eu^{3+}-release cytotoxicity assay of B6 Ad5E1B-specific CTL clone 01 (*A*), B6 CTL clone 21 (*B*), and B6 Ad5E1A-specific CTL clone 5 (*C*) on untransformed B6 MEC, B6 Ad5E1 MEC, and B6 Ad5E1 MEC expressing EJ*ras* at different effector-to-target cell ratios is shown.

Ad5E1B-specific CTL do not kill Ad5E1 + ras-transformed cells

The activated *ras* oncogene has been strongly implicated in the development of human malignancies (47, 48). Furthermore, experiments in a rat modelsystem have shown that Ad5E1-transformed cells expressing an activated *ras* oncogene, are highly oncogenic in immunocompetent syngeneic animals (49). To create a model for immunotherapy and vaccination strategies in immunocompetent animals we investigated the effect of an activated *ras* oncogene on the tumorigenicity of Ad5E1-transformed MEC in immunocompetent animals. Therefore, we generated cell lines tranformed by Ad5E1 and the EJ*ras* oncogene. Interestingly, several of these cell lines were tumorigenic both in nude and immunocompetent animals, despite the fact that they harbor coding sequences for at least two CTL epitopes (A. R. Offring, R. E. M. Toes, R. J. J. Blom, C. J. M. Melief, and W. M. Kast, unpublished results). This suggests that Ad5E1 + *ras* MEC evade destruction by the T cell-mediated immune response. B6 CTL clone 5, directed against Ad5E1A, and Ad5E1B-specific CTL clones 01 and 21 were tested for their ability to lyse B6 MEC transformed by Ad5E1 + EJ*ras* (Ad5E1 + *ras* MEC). Interestingly, these target cells were only lysed by Ad5E1A-specific CTL clone 5, but not by the Ad5E1B-specific CTL clones (Fig. 6). The observation that Ad5E1 + *ras*-transformed tumor cells are not lysed by Ad5E1B-specific CTL cannot be explained by a decrease of MHC class I expression on Ad5E1 + *ras*-transformed cells, because Ad5E1 + *ras* MEC and Ad5E1 cells have similar MHC class I expression levels (Fig. 7). The failure of Ad5E1B-specific CTL to lyse Ad5E1 + *ras* cells is also not attributable to an overall resistance to CTL-mediated cytotoxicity, because addition of the synthetic Ad5E1B-encoded peptide VNIRNCCYI to Ad5E1 + *ras* MEC resulted in lysis by Ad5E1B-specific CTL (data not shown). To demonstrate that the resistance of Ad5E1 + *ras* MEC to killing by Ad5E1B-specific CTL was caused by the action of the EJ*ras* oncogene, rather than by differences between individual cell lines, Ad5E1(wt) MEC and Ad5E1(P→S) MEC were supertransfected with the EJ*ras* oncogene. These supertransfected cells were not lysed by CTL clones 01 and 21, whereas Ad5E1(wt) MEC and Ad5E1(P→S) MEC supertransfected only with the hygromycine resistance gene were still efficiently lysed by the CTL clones (Table II). Although the Ad5E1B-protein expression varied between the cell lines, there is no correlation between Ad5E1B-protein levels and sensitivity to lysis by Ad5E1B-specific CTL: cell lines expressing a relatively

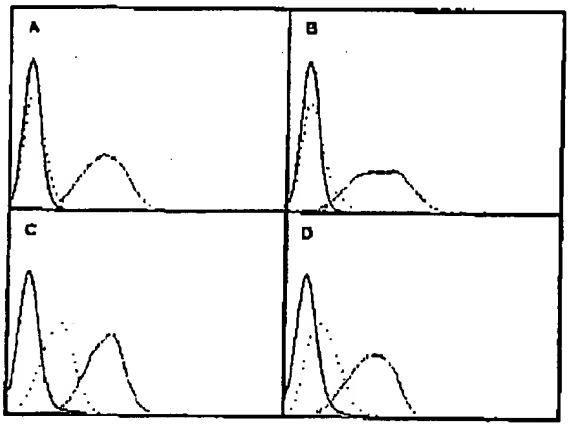

FIGURE 7. MHC class I expression levels of Ad5E1(wt) MEC and Ad5E1 + *ras* MEC as determined by FACS-analysis. H-2K^b (*panel A*) and H-2D^b (*panel B*) expression of Ad5E1(wt) MEC and H-2K^b (*panel C*) and H-2D^b (*panel D*) expression of Ad5E1 + *ras* MEC. —— = fluorescein isothiocyanate-labeled goat anti-mouse second Ab only; ··· = MHC class I expression after culturing the cells without IFN-γ; ····· = MHC class I expression after culturing the cells with IFN-γ. MHC class I expression levels after culturing the cells with IFN-γ are given, because all target cells used in a cell-mediated lymphocyte cytotoxicity assay were treated for 2 days with 10 U IFN-γ before use in the cell-mediated lymphocyte cytotoxicity assay.

Table II. *Lysis of B6 Ad5E1(wt) and B6 Ad5E1 (P→S) cells by Ad5E1B-, but not by Ad5E1A-, specific CTL clones is abolished by supertransfected of the activated EJras oncogene*[a]

	MEC	5E1(T→I)	5E1(P→S)	5E1(wt)	5E1(wt) hygr.	5E1(wt) hygr.+*ras* A	5E1(wt) hygr.+ras B	5E1(P→S) hygr.	5E1(P→S) hygr.+*ras*
CTL clone 5	0	100	−10	100	88	87	112	3	6
anti Ad5E1(wt)	0	97	3	96	71	82	94	3	9
	0	67	5	78	45	52	84	3	5
CTL clone 01	0	90	90	91	71	7	15	85	3
anti Ad5E1(T→I)	0	79	75	74	58	3	14	67	7
	−1	58	57	72	49	−9	10	46	3
CTL clone 21	0	94	100	97	53	−1	11	92	8
anti Ad5E1(P→S)	0	96	105	93	55	−6	10	88	10
	0	87	94	81	48	−10	5	73	10

[a] Lyde activity of B6 Ad5E1A-specific CTL clone 5 and B6 Ad5E1B-specific CTL clones 01 and 21 on B6 MEC, B6 Ad5E1(T→I) MEC, B6 Ad5E1(P→S) MEC, B6 Ad5E1(wt) MEC, B6 Ad5E1(wt) MEC supertransfected with only the hygromycine resistance gene, B6 Ad5E1(wt) MEC supertransfected with the hygromycine resistance gene and EJ*ras* (two independent clones A and B), B6 AdE1(P→S) MEC supertransfected with only the hygromycine resistance gene and B6 Ad5E1(P→S) MEC supertransfected with the hygromycine resistance gene and EJ*ras*. Expression of EJras was confirmed at the protein level by Western blotting with the use of mAb Y13-259 (50). Percentage of specific lysis as determined by an Eu^{3+}-release cytotoxicity assay at effector-to-target cell ratios of 10, 5, and 2.5 are shown.

low level of Ad5E1B protein (see Figure 8, *lanes 3* or *8*) are lysed, whereas cell lines expressing a higher level of Ad5E1B (see Figure 8, *lanes 6* and *7*) are not lysed by Ad5E1B-specific CTL clones (Table II). Also the MHC class I expression cannot explain the insensitivity of Ad5E1(wt) + *ras* or Ad5E1(P→S) + *ras* MEC by Ad5E1B-specific CTL clones, because these cells express considerable levels of MHC class I (Fig. 9). Furthermore, (super)transfection of Ad5E1(wt) MEC with EJ*ras* did not abolish recognition by the Ad5E1A-specific CTL (Fig. 6; Table II), suggesting that the EJ*ras* oncogene selectively influences the presentation of the Ad5E1B-encoded CTL epitope by the tumor cells.

Efficient tumor eradication in vivo by CTL generated against mutant Ad5E1 MEC

In a previous report we demonstrated that Ad5E1-induced tumors in B6 nude mice can be efficiently eradicated by a single injection of B6 Ad5E1A-specific CTL clone 5, provided that rIL-2 was given simultaneously (10). To test whether CTL clones directed against the Ad5E1B-encoded CTL epitope can also destroy tumors in vivo, B6 nude mice were inoculated with 10^7 Ad5E1(wt) MEC. Two weeks later, at the time when all mice had developed tumors, these mice were treated with B6 Ad5E1B-specific CTL clone 01 or 21 in combination with rIL-2. Both CTL clones were, like the Ad5E1A-specific CTL clone 5, capable of efficient tumor eradication (Fig. 10), indicating that CTL activity directed against different CTL epitopes expressed by the same tumor can serve as a potent tool for tumor-destruction in vivo.

Discussion

B6 Ad5E1(wt) MEC present at least two CTL epitopes to the immune system. One CTL epitope is encoded by Ad5E1A, whereas the second CTL epitope is located on another viral gene product, namely the 55-kDa Ad5E1B protein. The observation that the T cell immune response is directed to more than one adenovirus encoded gene-product might have important implications for adenovirus-based in vivo gene therapy, as it has been described that an adenovirus-specific cellular immune response can severely hamper the effectiveness of adenovirus-based gene therapy (54). One approach for improving recombinant adenoviruses for gene therapy is based on crippling the virus to limit the expression of viral genes to prevent a virus-specific cellular immune response. The observation, even on one genetic background, that Ad5E1-transformed tumor cells express at least two CTL epitopes that can mediate protective immunity, might severely limit such an approach. This is especially true if the approach is designed for an outbred (human) population.

Previously, we showed that ten out of ten independently derived CTL clones generated against Ad5E1(wt) MEC recognized the Ad5E1A-encoded CTL epitope SGPSNT PPEI (10), suggesting the immunodominance of this epitope in the Ad5E1-specific immune response. Data reported by others (55) supported this notion, since immunization of H-2^b mice with whole Ad5 virus induced an H-$2D^b$-restricted T cell response that was directed against Ad5E1A, despite the expression of a variety of other viral proteins by the infected cells. The Ad5E1A gene encodes proteins that also seem to be immunodominant in the context of other MHC class I molecules (56, 57), although Ad5E1A is not a major target Ag for Ad5-specific CTL in either BALB/c (H-2^d) or C3H/HeJ (H-2^k) mice (55). We now demonstrate that mice expressing H-2^b are also able to mount a strong CTL response directed against a second, Ad5E1B-encoded epitope. At first, we only observed the Ad5E1B-specific response after immunization of mice with mutant Ad5E1 MEC that harbor a mutation in the Ad5E1A-encoded epitope (Fig. 3). Further experimentation, however, revealed that this specificity could also be raised by immunizing mice with Ad5E1(wt) MEC (data

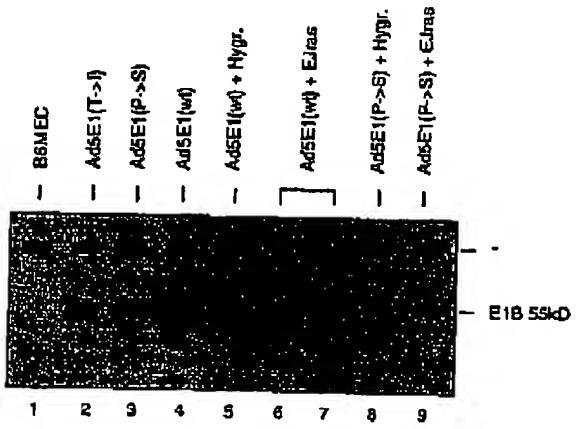

FIGURE 8. Ad5E1B-protein expression levels in Ad5E1-transformed cell lines. Equivalent amounts of cell lysates were assayed by Western blotting essentially as described by Harlow and Lane (51). The Ad5E1B protein was detected by mouse mAb A1C6 (52). *Lane 1*: B6 MEC; *lane 2*: B6 Ad5E1 (T→I) MEC; *lane 3*: B6 Ad5E1(P→S) MEC; *lane 4*: B6 Ad5E1(wt) MEC; *lane 5*: B6 Ad5E1(wt) MEC supertransfected with only the hygromycine resistance gene; lanes *6* and *7*: B6 Ad5E1(wt) MEC supertransfected with the hygromycine resistance gene and *EJras* (two independent clones; *A* and *B*), *lane 8*: B6 Ad5E1(P→S) MEC supertransfected with only the hygromycine resistance gene; *lane 9*: B6 Ad5E1(P→S) MEC supertransfected with the hygromycine resistance gene and *EJras*. Protein bands marked by * represent nonspecific background, because these bands were also present after incubation of the blot with goat anti-mouse Abs and, subsequently, streptavidin/horseradish peroxidase and the chemiluminiscence reagents ("second step only") (53). These background bands were used as a reference for protein amounts loaded. The relative Ad5E1B-expression in the different cell lines was quantitated after densitometric analysis by calculating the ratio: Ad5E1B-signal/("second step only"-signal and Ad5E1B-signal). *Lane 1*: 0, *lane 2*: 0.65, *lane 3*: 0.40, *lane 4*: 0.80, *lane 5*: 0.67, *lane 6*: 0.60; *lane 7*: 0.48, *lane 8*: 0.17, *lane 9*: 0.18.

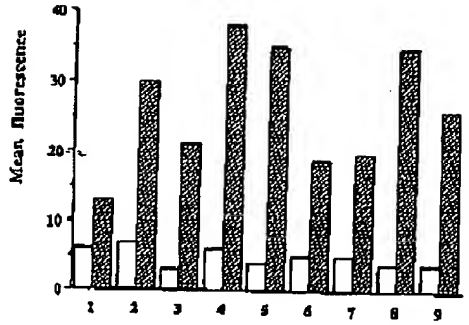

FIGURE 9. H-2D^b-expression levels of the tumor cell lines listed in Table II. 1) B6 MEC; 2) B6 Ad5E1(T→I) MEC; 3) B6 Ad5E1(P→S) MEC; 4) B6 Ad5E1(wt) MEC; 5) B6 Ad5E1(wt) MEC supertransfected with only the hygromycine resistance gene; 6) and 7) B6 Ad5E1(wt) MEC supertransfected with the hygromycine resistance gene and *EJras* (two independent clones; *A* and *B*); 8) B6 Ad5E1(P→S) MEC supertransfected with only the hygromycine resistance gene; 9) B6 Ad5E1 (P→S) MEC supertransfected with the hygromycine resistance gene and *EJras*. H-2D^b expression levels are shown after culturing the cells for 2 days with 10 U IFN-γ (see also figure 7.). White bars: fluorescein isothiocyanate-labeled goat anti-mouse second Ab only; dashed bars: mAb specific for H-2D^b (41) and a fluorescein isothiocyanate-labeled goat anti-mouse Ab.

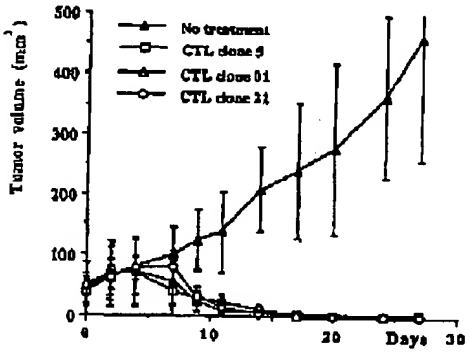

FIGURE 10. Treatment of B6 nude mice carrying a subcutaneous B6 Ad5E1 tumor. B6 nude mice carrying Ad5E1(wt)-induced tumors were left untreated (closed triangles), or were treated at day zero by i.v. injection of 1.5×10^7 B6 AdrE1A-specific CTL clone 5 (open squares), 1.5×10^7 B6 Ad5E1B-specific CTL clone 01 (open trinagles), or 1.5×10^7 B6 Ad5E1B-specific CTL clone 21 (open circles) in combination with 2.5×10^5 Cetus units of rIL-2 in IFA.

not shown). Thus, Ad5E1(wt) tumor cells are able to induce a CTL response against both the Ad5E1A- and Ad5E1B-encoded CTL epitopes.

B6 mice not only have the ability to raise immunity against each of these epitopes, but also CTL directed against either of these epitopes can eradicate established tumors upon adoptive transfer (Fig. 10). The latter observation holds promise for the immunotherapeutic treatment of malignancies. Several studies of both virally and non-virally induced tumors have shown that tumor cells can, like viruses, escape from destruction through mutation or down-modulation of T cell epitopes. (13, 15–17, 58–63). In the treatment and prevention of malignancies, it might therefore be important to direct the immune response to several T cell epitopes, thereby reducing the risk of tumor cell escapes from immune surveillance via Ag loss or mutation. Results from studies with human tumors imply that such a clinical approach is indeed feasible. Multiple tumor Ags have, for instance, been identified in human melanoma (18–24).

Finally, our data also reveal a potential complication of cancer immunotherapy. Ad5E1 + *ras* MEC that contain

an activated H-*ras* oncogene (EJ*ras*), in addition to the Ad5E1-transforming genes, do not show sensitivity to lysis by Ad5E1B-specific CTL (Fig. 6; Table II). The failure of the Ad5E1B-specific CTL to lyse these tumor cells does not involve insufficient protein expression levels of either MHC class I or Ad5E1B (Figs. 7–9). Furthermore, Ad5E1 + *ras* MEC do not display an overall resistance to CTL-mediated lysis, because these cells are efficiently killed by Ad5E1A-specific CTL (Fig. 6; Table II). Taken together, these data suggest that in EJ*ras*-expressing tumor cells, the presentation of the Ad5E1B epitope is affected. Apparently, the EJ*ras*-induced transformation process somehow interferes with the processing of the Ad5E1B protein, while not detectably altering the processing of the Ad5E1A gene product. We are testing whether transformation by EJ*ras* may alter the presentation of additional T cell epitopes. Because activated *ras* oncogenes have been strongly implicated in the development of human cancer, modulation of T cell epitopes by a *ras*-induced mechanism may be instrumental in the failure of T cell immunity against malignant tumors in humans (47, 48).

In conclusion, we show that the immune response against different T cell epitopes can be employed in tumor-specific immunotherapy. Down-modulation of some of the epitopes involved may, however, compromise the effectiveness of the immunotherapy. The latter observation stresses the importance of directing the anti-tumor immune response to multiple T cell epitopes, thereby minimizing the chance of T cell immune response evasion by tumor cells.

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